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Fibronectin induces ureteric bud cells branching and cellular cord and tubule formation

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Fibronectin induces ureteric bud cells branching and cellular cord and tubule formation.

Background. The extracellular matrix (ECM) protein fibronectin is involved in several stages of embryogenesis. Fibronectin exerts its effect through interaction with cellular integrin and nonintegrin receptors.

Methods. We investigated the effect of fibronectin on branching and tubulogenesis of ureteric bud cells in a three-dimensional gel culture system. Primary ureteric bud cells from mouse embryos at gestation 11 days (E11) were isolated and established in culture. Fibronectin and integrin subunits were localized using immunoperoxidase staining.

Results. In three-dimensional collagen type I gel culture of ureteric bud cell, fibronectin dose dependently induces cord and tubule formation. Both ureteric bud cells and ureteric bud branches in embryonic kidney express the same multiple integrin subunits that include β_1, β_3, α_3, α_4 and α_v. Embryonic kidneys examined at E12, E14, and E16 days of gestation express fibronectin in the undifferentiated mesenchyme especially next to ureteric bud branches and in the interstitium around glomerulotubular structures and blood vessels. Fibronectin expression was similar at the tips and stalks of branching ureteric bud. Fibronectin expression is maximum at E12 and decreases with advanced gestation. Cultured ureteric bud cells also express fibronectin. RGD peptides inhibit cord and tubular formation in the three-dimensional gel. Anti-$\alpha_3\beta_1$ antibody partially inhibits fibronectin-induced cord and tubule formation. Hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and glial cell line-derived neurotrophic factor (GDNF) induce ureteric bud cell cord formation in three-dimensional gel. The effects of growth factors are delayed and quantitatively less compared to the effect of fibronectin.

Conclusion. Fibronectin induces ureteric bud cells branching and tubulogenesis through interaction with multiple integrin receptors. Cultured ureteric bud cells express febronectin and the origin of fibronectin at mesenchyme-ureteric bud interface is likely both the metanephric mesenchyme and ureteric bud epithelium. Addition of individual neutralizing antibodies to β_1, β_3, α_3, α_4, α_6 and α_v integrin subunits does not block the effect of fibronectin. Only an antibody to $\alpha_3\beta_1$ integrin substantially blocks the effect of fibronectin. Other mechanisms, including unidentified integrins, are likely involved in fibronectin-induced cord and tubule formation.

Key words: integrin receptor, three-dimensional gel, extracellular matrix.

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Development of mature functioning kidney depends on inductive interactions between the metanephric mesenchyme and the ureteric bud [1–3]. Renal organogenesis begins when the ureteric bud, an outgrowth of the Wolfian duct, reciprocally interacts with metanephric mesenchyme. This interaction, acting through various signals, causes the ureteric bud to grow, branch and differentiate to form the urinary collecting duct system [4–7]. Branching morphogenesis depends on interactions between different soluble factors and extracellular matrix (ECM) components with tubular cell precursors [8]. Fibronectins are multifunctional ECM glycoproteins that interact with other ECM components such as collagen, fibrin, heparin, and hyaluronic acid. Fibronectin also interacts with integrin receptors on the cell surface [9–11]. Fibronectins participate in several aspects of cell behavior [12, 13]. These biologic responses are relevant to development, wound healing, and tumorigenesis [14]. Targeted null mutation in the genes for fibronectin [15], and for either of its two major integrin receptors, display distinct phenotypes, and they are lethal early in development.

ECM components such as fibronectin and laminin have been shown to stimulate tubulogenesis, while other components such as tenasin are inhibitory. Hepatocyte growth factor (HGF)–transfected mouse inner medullary collecting duct (IMCD-3) cells form branching tubules when grown in three-dimensional collagen gel. Expression of HGF and c-met in these renal epithelial cells stimulates fibronectin gene expression [16].

Fibronectin is a glycoprotein consisting of repeated units of amino acids, which form domains that enable the molecule to interact with a variety of cells through both integrin and nonintegrin receptors. It is encoded

by a single gene, but alternative splicing of pre-mRNA allows formation of multiple isoforms that have critical roles in cell adhesion, migration, differentiation, and proliferation [13, 15]. It is now clear that fibronectin influences a variety of cellular processes which subsequently modulate signal transduction within cells. A recently published study demonstrated a critical role of fibronectin in branching morphogenesis of salivary gland, lung, and kidney. This study suggested a larger role of ureteric bud elongation than branching in kidney morphogenesis. However, the same study stressed the increased relative concentration of fibronectin at sites of epithelial constriction and indentation [17]. Understanding the in vivo role of fibronectin remains incomplete. Many integrins act as fibronectin receptors in numerous cell types. These integrins include $\alpha_5\beta_1, \alpha_4\beta_1, \alpha_3\beta_1, \alpha_v\beta_1, \alpha_v\beta_3$, and $\alpha_v\beta_6$. Among these integrins, $\alpha_3\beta_1$ and $\alpha_v\beta_1$ have been described in the developing ureteric bud.

Very little is known about the effect of fibronectin on ureteric bud cell tubulogenesis. In this study we show that fibronectin induces ureteric bud cell tubulogenesis in three-dimensional gel culture system and this effect is mediated via integrin receptors. $\alpha_3\beta_1$ integrin receptor is involved in mediating fibronectin effect. Fibroblast growth factor (FGF), glial cell line-derived neurotrophic factor (GDNF), and HGF also cause ureteric bud cell tubulogenesis in three-dimensional gel. HGF and FGF effects on ureteric bud cell cord and tubule formation are additive to the effect of fibronectin.

METHODS

Materials

Rat antihuman integrin α_6 (MAB 1378), rabbit anti-integrin α_V subunit (AB1930), all antimouse integrin antibodies were purchased from Pharmingen (San Diego, CA, USA), mouse antihuman integrin $\alpha_3\beta_1$ (MAB 1992) was purchased from Chemicon (Temecula, CA, USA). Mouse antivimentin (814 318) was purchased from Boehringer Mannheim (Indianapolis, IN, USA). Rhodamine *Dolichus biflorus* (RL-1032) was purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). Fluorescein isothiocyanate (FITC)-labeled *Lotus tetragonolobus* agglutinin (LTA) lectin (L5644), and mouse monoclonal anticellular fibronectin (F6140) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Avidin-biotin complex (ABC) reagents were purchased from Vector Staining Laboratories, Inc. (Burlingame, CA, USA), murine fibronectin (12173–019) was obtained from Invitrogen, Life Technologies, (Carlsbad, CA, USA). Rat tail type I collagen used to prepare three-dimensional gel was obtained from Collaborative Biochemical. RGD neutralizing peptide (Gly-Arg-Gly-Asp-Ser, G4391) and control peptide (Ser-Asp-Gly-Arg-Gly, S 3771) were obtained from Sigma (St. Louis, MO, USA). FGF, GDNF, and HGF were purchased from R&D Systems Inc. (MN).

Animals

Pregnant mice were purchased at gestation day 10 (E10) from Charles River Laboratories (Wilmington, MA, USA). The age of the embryo was counted from the day of the vaginal plug (day 0). Mice were anesthetized by intramuscular injection of mice cocktail (60% ketamine, 40% xylazine, 0.1 mL/100 g) and embryos were collected. Embryos were dissected in 1 × phosphate-buffered saline (PBS) under a zoom model SZH Olympus stereomicroscope and embryonic kidneys were obtained. Ureteric bud was dissected at gestation day 11 or 12 and processed for cell culture or the whole embryonic kidney was placed in optimal cutting temperature (OCT) compound and frozen in liquid nitrogen. Embryonic kidneys were stored at −70°C until cryosectioning.

Immunohistochemistry and immunofluorescence

Embryonic kidneys were isolated and stored at −70°C. Frozen tissue was cut and prepared for indirect immunoperoxidase amplified by the ABC technique [3, 18–20]. Sections were incubated with 0.6% hydrogen peroxide in methanol to block nonspecific peroxidase activity and 0.01% avidin 0.001% biotin to block localization of endogenous biotin activity and incubated with normal serum of the same species as the second antibody to block nonspecific antibody staining. Antibodies to fibronectin and anti-integrin subunits antibodies were applied followed by biotin-labeled secondary antibodies with appropriate washes in between. Antibody to fibronectin is a monoclonal anticellular fibronectin that localizes the 240 kD bands of cellular fibronectin. The integrin subunits that were explored include β_1, β_3, β_4, α_3, α_4, α_5, and α_v. Staining was developed utilizing the ABC reagents and methods supplied by the manufacturer (Vector Laboratories, Inc.). Direct and indirect immunofluorescence staining was also used to examine the expression of vimentin, LTA, and *Dolichus biflorus* (rhodanine-conjugated lectin specific for ureteric bud). Controls consist of nonimmune IgG of the appropriate species of primary antibody or dilutent without primary antibody.

Preparation and isolation of ureteric bud cells

Ureteric bud was isolated from 11 or 12 day mouse embryonic kidney and tissue was collected from embryos of two pregnant mice. The cells were mechanically dissociated by gentle aspiration with a pipette. The resulting single cell suspension was pelleted by low-speed centrifugation (1500 rpm) for 5 minutes, resuspended and plated on costar tissue culture dishes in 10% fetal bovine serum (FBS) Dulbecco's modified Eagle's medium (DMEM).

Cells were grown at 37°C in 5% CO_2 to 60% to 70% confluence and transformed with conditioned media from PA 317 LXNX 16 E6/E7 cells that contain human papilloma virus for immortalization. Cells express *Dolichus biflorus* (specific lectin that bind to ureteric bud), cytokeratin (epithelial intracellular filaments), and LTA (lectin that bind to both ureteric bud and proximal tubule) and are negative for vimentin (mesenchymal intracellular filaments).

Three-dimensional gel culture

Three-dimensional gels are prepared as described previously with some modifications [21–23]. Collagen gels are prepared by mixing eight parts (400 μL) of sterile collagen solution [rat tail collagen, type I (3.43 mg/mL)], with one part (50 μL) of 0.2 mol/L Hepes, 10 μL of 1 N NaOH, and 450 μL of 2× DMEM 20% fetal calf serum (FCS). The gel mix was placed on ice to prevent gelling. Then, 25 μL of gel mix were transferred to the bottom of each well of an 8 chamber slide, allowed to spread evenly, and incubated at 37°C for 30 minutes. A collagen/cells gel was prepared as above with added ureteric bud derived cells to the DMEM/FBS at a final concentration of 5×10^4 cells/mL. Fibronectin was added to the gel at concentrations between 10 and 100 μg/mL. The collagen/cell solution was transferred to the wells (100 μL/well) and allowed to gel at 37°C for 30 minutes. The gel was then covered with 500 μL of 10% FBS DMEM. Inhibitory RGD peptides were added at a concentration of 1 mg/mL. Integrin subunits antibodies were added to cell suspension at the concentration of 8 μg/mL. To test the effect of different growth factors on ureteric bud cells, HGF (50 μg/mL), GDNF (5 μg/mL), and FGF (25 μg/mL) were added to the ureteric bud cell culture in three-dimensional gel with and without fibronectin (50 μg/mL). Ureteric bud cells cord formation was assessed in each well.

Verification of tubule formation by nuclear staining and diffraction interference contrast microscopy

Ureteric bud cells were grown in three-dimensional gel with and without fibronectin for 4 days. The ureteric bud cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature and washed extensively with PBS. The fixed ureteric bud cells were stained with ethidium bromide (50 mg/mL) for 10 minutes at room temperature. Cells were washed 6 times with PBS to remove the excess staining. The photographs were taken using digital camera attached to a Nikon Eclipse TE300 inverted microscope. Each assay was performed at least in triplicate.

Three-dimensional gel containing branching ureteric bud cells grown for 4 days was placed in the cryomold, covered with freezing medium (OCT) then frozen in isopentane at −48°C. Frozen sections were cut at 5 μ thickness and placed on glass slides. Diffraction interference contrast images were obtained using an Olympus AX70 research microscope (Melville, NY, USA).

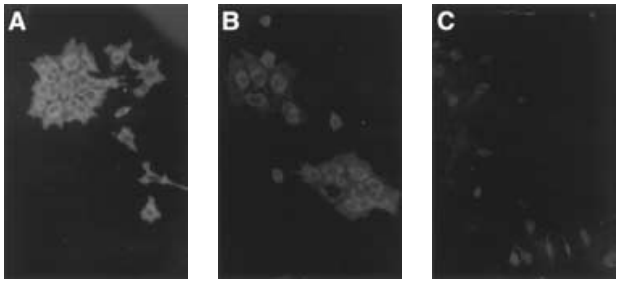

Fig. 1. Mouse ureteric bud cells. Ureteric bud was dissected at 11 days' gestation. Cells were dissociated and established by transfection with human papilloma virus. Cells were plated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). (*A*) Immunofluorescence localization of *Dolichus biflorus* in ureteric bud cells after 3 days in culture. (*B*) Immunofluorescence localization of *Lotus tetragonolobus* agglutinin (LTA) lectin in ureteric bud cells. (*C*) Immunofluorescence localization of vimentin in ureteric bud cells.

Statistical analysis

Quantitative data of cellular cord formation are expressed as mean ± SD. Unpaired, two-tailed *t* test was used to compare the effects of fibronectin-containing gel vs. collagen type I gel without fibronectin and the effects of added HGF, GDNF, and FGF to the three-dimensional gel vs. absence of these growth factors. Significance was accepted at $P < 0.05$.

RESULTS

Characterization of ureteric bud cells

Ureteric bud cells were isolated and cultured as described in the **Methods** section. These cells were propagated in culture up to passage 25. Figure 1A shows immunofluorescence localization of *Dolichus biflorus*, a specific ureteric bud marker. Ureteric bud cells also express LTA (lectin that is expressed by both ureteric bud and proximal tubule) (Fig. 1B) and are negative for vimentin, a mesenchymal intracellular filament (Fig. 1C).

Localization of fibronectin

To study the expression of fibronectin in developing kidney, immunoperoxidase staining was performed using monoclonal anticellular fibronectin-specific antibody. Figure 2 shows diffuse expression of fibronectin throughout the mesenchyme with increased concentration surrounding the developing glomerulotubular structures, ureteric bud branches, and vessels. No fibronectin expression was noted in the developed ureteric bud epithelial cells. However, cultured ureteric bud cells express fibronectin (Fig. 3). Fibronectin expression decreases with maturation of embryonic kidney and the main expression was seen around blood vessels at later stages of

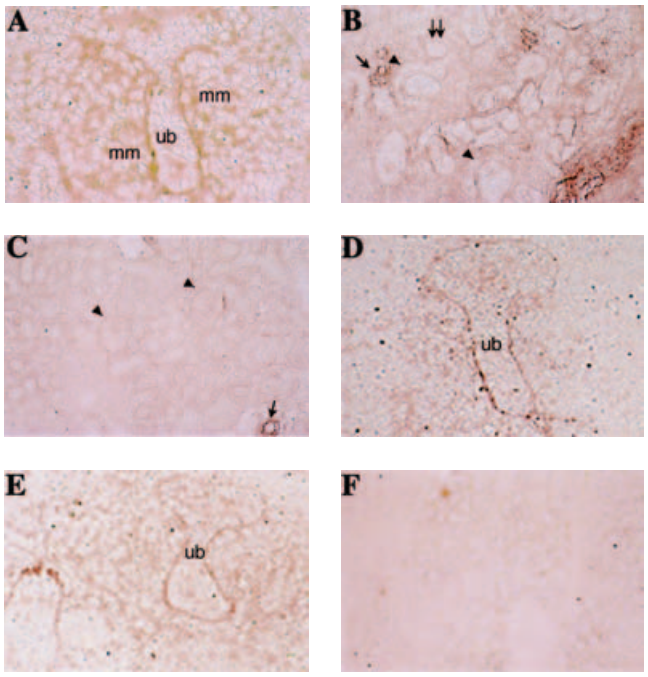

Fig. 2. Immunohistochemical localization of fibronectin in 12 (*A*), 14 (*B*), and 16 (*C*) day mouse embryonic kidney. Fibronectin is expressed in the undifferentiated metanephric mesenchyme (mm) and concentrated around ureteric bud (ub) at 12 days' gestation. Fibronectin expression decreases at later time points and it is localized in the interstitium surrounding glomerulotubular structures, ureteric bud branches, and vessels. No fibronectin expression was noted in the differentiated epithelial cells. (*D* and *E*) Additional sections from 12 day embryonic kidneys that show fibronectin is equally expressed at the tips and stalks of branching ureteric bud. (*F*) Negative control. Symbols are: ↓↓, ureteric bud branches; ►, glomerulotubular structures; ↓, blood vessel.

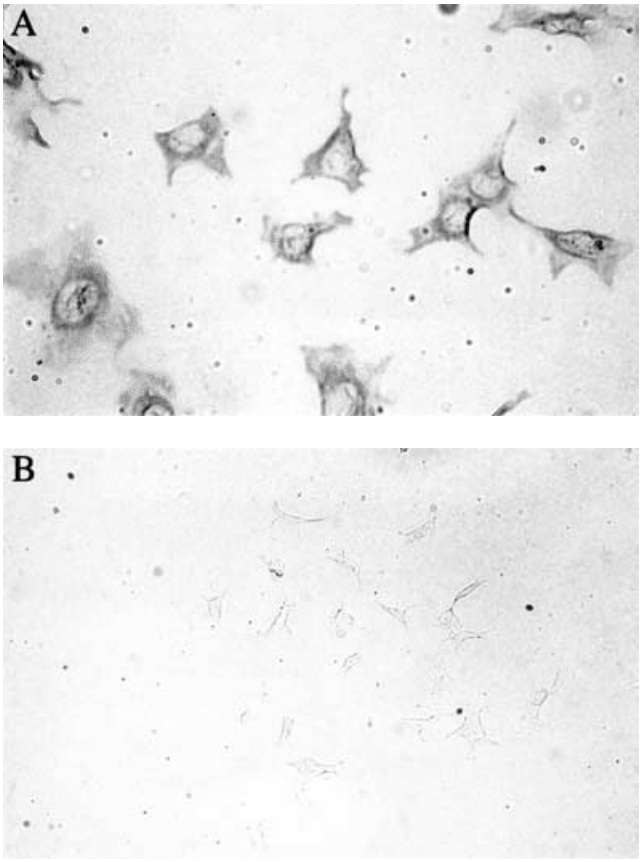

Fig. 3. Immunohistochemical localization. (*A*) Fibronectin in cultured ureteric bud cells. (*B*) Negative control.

development. Fibronectin expression appears uniform at the tips and stalks of branching ureteric bud (Fig. 2A, D, and E).

Localization of integrin subunits in embryonic kidney and ureteric bud cells

To study the expression of integrin subunits in developing kidney and ureteric bud cells, immunoperoxidase staining was performed using specific anti-integrin subunits antibodies. Figure 4 shows localization of integrin subunits in 14-day embryonic kidney and Figure 5 shows localization of integrin subunits in ureteric bud cells. Both developing ureteric bud and transformed ureteric bud cells express α_3, α_4, α_v, β_1, and β_3 subunits but not β_4 or α_5.

Formation of cellular cords and tubules in three-dimensional gel culture

Ureteric bud cells grown in collagen type I three-dimensional gel form cellular aggregates and cystic-like structures. Addition of fibronectin to the collagen gel induces cellular elongation and process formation. Cells begin to migrate from cellular aggregate and eventually form cords and tubules in response to increasing concentration of fibronectin (Fig. 6). Ethidium bromide nuclear staining of ureteric bud cell cords and tubules is shown in Figure 7. Diffraction interference contrast microscopy of three-dimensional gel sections show that fibronectin induces formation of tubules with lumens (Fig. 8).

Effect of growth factors on ureteric bud cell cord and tubule formation

HGF, FGF, and GDNF induce ureteric bud cell cord formation in collagen I three-dimensional gel. Effects of both FGF and GDNF occur after 4 days in culture while HGF effect is significant after 2 days (data not shown). Fibronectin effect is many folds higher than any of the growth factors and is noticed after 24 hours. It is interesting that the effect of fibronectin and either of HGF or FGF are additive. Although the GDNF and fibronectin effects appear to be additive. However, it did not achieve statistical significance (Table 1).

Inhibition of fibronectin-induced cord/tubule formation of ureteric bud cells by inhibitory RGD peptides

Addition of RGD inhibitory peptide Gly-Arg-Gly-Asp-Ser to the three-dimensional gel prevents the

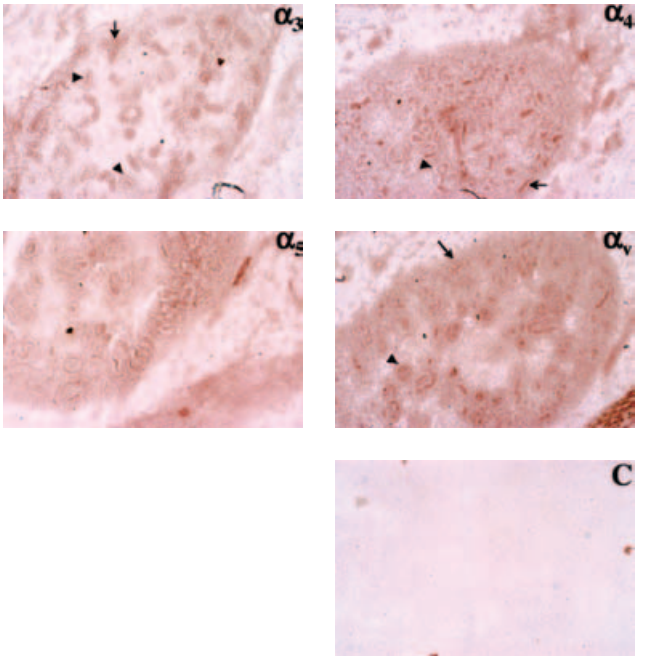

Fig. 4. Immunohistochemical localization of selected integrin alpha subnunits in 14 day embryonic kidney. α_3, α_4, α_5, and α_v, are known to act as fibronectin receptors in different cell types. α_3, α_4, and α_v are expressed in the ureteric bud branches. α_5 subunit is absent. β_1 and β_3 integrin subunits (not shown) are also expressed by the ureteric bud. Negative control (C) uses nonimmune IgG of the same species as that of the primary antibody. Symbols are: ↓, ureteric bud branches; ►, glomerulotubular structures.

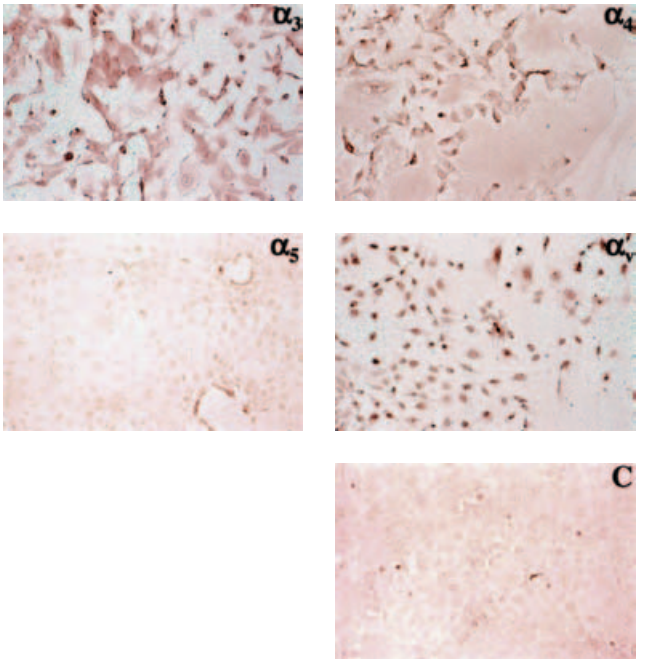

Fig. 5. Immunohistochemical localization of selected integrin alpha subunits in immortalized cells derived from mouse ureteric bud. These cells express α_3, α_4, and α_v integrin subunits which are known to act as receptors for fibronectin. α_5 subunit is absent. These cells also express β_1 and β_3 integrin subunits (not shown). Negative control (C) uses nonimmune IgG of the same species as that of the primary antibody.

formation of cords and tubules. Ureteric bud cells form cellular aggregates similar to those grown in three-dimensional gel that contains only type I collagen with no added fibronectin (Fig. 9).

Anti-integrin $\alpha_3\beta_1$ partially inhibits the ureteric bud cell branching and cord/tubule formation

The three-dimensional gel culture was treated with anti-integrin subunits at the time of addition of ureteric bud cells to the gel. Only antibodies to a $\alpha_3\beta_1$ partially inhibited fibronectin-induced cord and tubule formation (Fig. 10). Antibodies against α_3, α_4, α_v, β_1, and β_3 failed to inhibit fibronectin effect. Partial inhibition of ureteric bud cell branching and cord/tubule formation suggest that fibronectin effect on ureteric bud cells is mediated in part through integrin $\alpha_3\beta_1$ and other unknown integrins or other nonintegrin signaling pathways mediate the fibronectin effect.

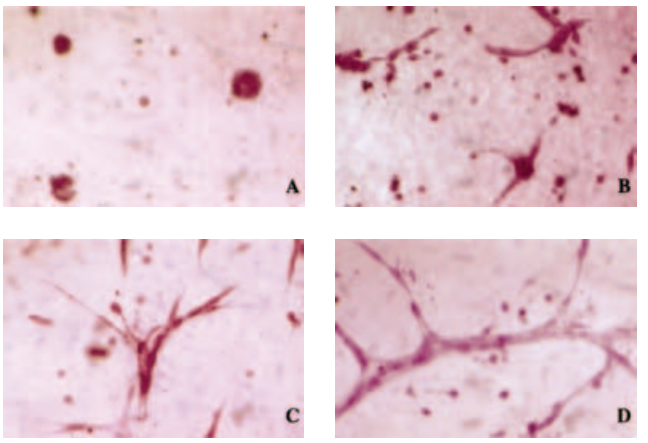

Fig. 6. Ureteric bud cells in three-dimensional gel culture. (*A*) Ureteric bud cells grown in collagen type I three-dimensional gel for 5 days show cellular aggregates and cyst-like structures. (*B*) Ureteric bud cells grown in three-dimensional gel as in (A) with added fibronectin (10 μg/mL) show cellular elongation and process formation (branching) with cells beginning to migrate from cellular aggregates to form cords. (*C*) Further development of cellular cords in three-dimensional gel containing 50 μg/mL fibronectin. (*D*) Three-dimensional gel containing 100 μg/mL fibronectin showed marked cellular cord/tubule formation.

DISCUSSION

Branching morphogenesis depends on interactions between different soluble factors and ECM components with tubular cell precursors. Fibronectins are multifunctional ECM glycoproteins that interact with other ECM components and integrin receptors on the cell surface [9–11]. Fibronectins participate in several aspects of cell behavior. These include adhesion, motility, differentiation and cytoskeletal reorganization [12, 13]. These cellular responses are essential for development.

We show that fibronectin induces cord and tubule formation of ureteric bud cells grown in three-dimensional

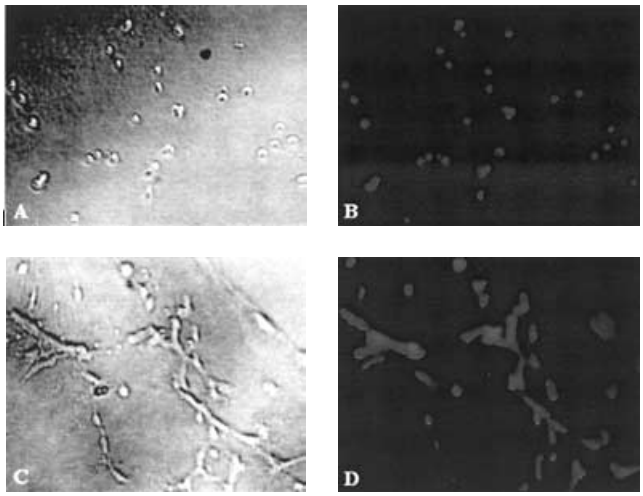

Fig. 7. Nuclear staining of ureteric bud cells. (*A*) Ureteric bud cells grown in collagen I three-dimensional gel. (*B*) Same three-dimensional gel shown in (A) stained with ethidium bromide. (*C*) Ureteric bud cells grown in fibronectin containing three-dimensional gel. (*D*) Same three-dimensional gel shown in (C) stained with ethidium bromide.

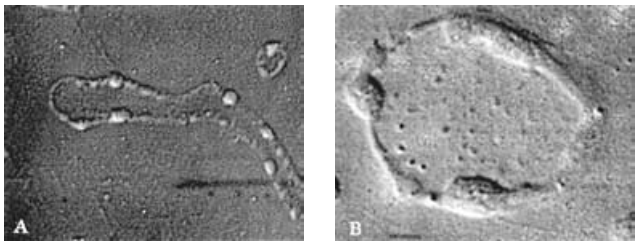

Fig. 8. Evidence of lumen formation in three-dimensional gel. Ureteric bud cell were grown in three-dimensional gel containing fibronectin for 4 days. (*A*) Diffraction interference contrast microscopy of 5 μ section from three-dimensional gel shows a longitudinal view of a tubule with lumen formation. A cross-section through another tubule is also shown. (*B*) Cross-section through a tubule at higher magnification.

Table 1. Effect of fibronectin, hepatocyte growth factor (HGF), glial cell line-derived neurotrophic factor (GDNF), and fibroblast growth factor (FGF) on ureteric bud cell cord formation after 4 days culture in three-dimensional gel

Experimental condition	Number of cellular cords (mean ± SD)	*P* value
Collagen type I	3.7 ± 1.5	
Collagen type I + fibronectin	585 ± 87	0.00001[a]
Collagen type I + HGF	12.7 ± 2.1	0.004[a]
Collagen type I + fibronectin + HGF	784 ± 85	0.002[b]
Collagen type I + GDNF	16 ± 4.6	0.011[a]
Collagen type I + fibronectin + GDNF	652 ± 50	0.17[b]
Collagen type I + FGF	13.3 ± 1.5	0.001[a]
Collagen type I + fibronectin + FGF	805 ± 101	0.009[b]

Cellular cord is defined as two or more ureteric bud cells connected by cellular processes with a cord or tubule configuration. 4×10^4 cells/mL were added to the gel.

[a]Significance compared to collagen type I; [b]Significance compared to collagen type I + fibronectin.

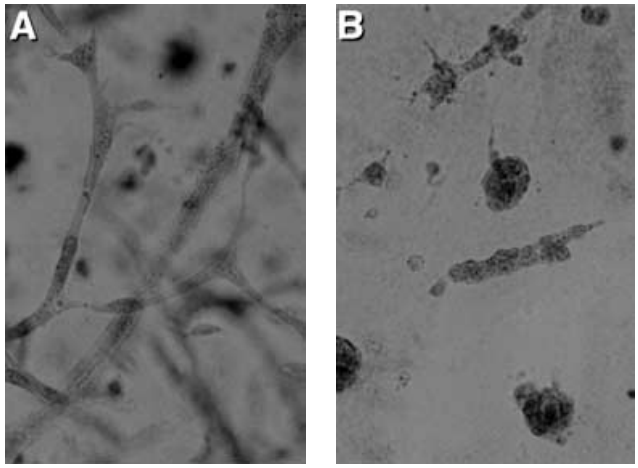

Fig. 9. Inhibitory RGD peptide (1 mg/mL) block fibronectin-induced cord/tubule formation of ureteric bud cells. (*A*) Ureteric bud cells grown in collagen type 1 and fibronectin. (*B*) Ureteric bud cells grown in three-dimensional gel as in (A) with added RGD peptide.

collagen type I gel culture. Branching morphogenesis of ureteric bud in vivo is likely influenced by fibronectin based on the distribution of fibronectin in the embryonic kidney. Fibronectin is expressed in the uninduced metanephric mesenchyme [26]. In this study we show that fibronectin expression is maximum early in nephrogenesis and decreases at later stages of renal development. Fibronectin expression is maximum at 12 days' gestation. At this stage, fibronectin is expressed in the nondifferentiated metanephric blastema with maximum expression next to branching ureteric bud. Fibronectin expression is equally distributed next to the tips and stalks of branching ureteric bud. Fibronectin expression decreases at 14 days' gestation with minimal expression at 16 days' gestation where it is mainly seen next to the developing vessels.

We also show that both isolated and cultured ureteric bud cells and in vivo developing ureteric bud express α_3, α_4, α_v, β_1, and β_3 integrin subunits. RGD peptides, that block integrin receptors interaction with ECM, completely inhibit the fibronectin-induced cord and tubule formation. Anti-$\alpha_3\beta_1$ partially inhibits fibronectin-induced cord and tubule formation. Ureteric bud cells treated with anti-$\alpha_3\beta_1$ exhibit some spreading and branching, an indication that other mechanisms yet unidentified are important in fibronectin-induced effect. It is likely that other integrin subunits or other mechanisms are also involved in the fibronectin effect since RGD peptides completely reversed fibronectin effect.

Previous investigations have shown important roles of fibronectin in cell adhesion, migration, differentiation, and proliferation [13, 15]. The essential role of fibronectin in fetal development has been shown in a knockout mouse model in which early lethality occurs

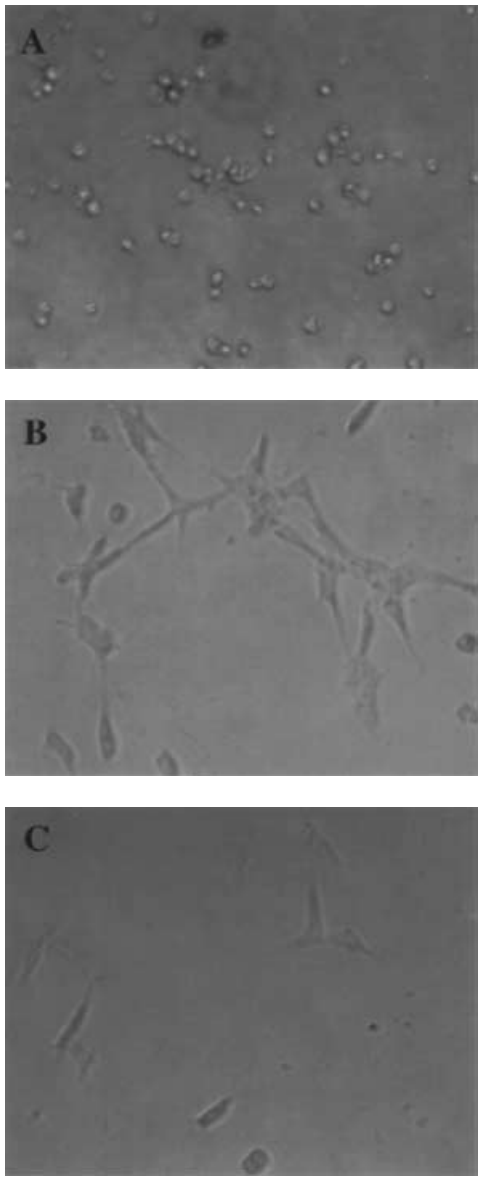

Fig. 10. Anti-$\alpha_3\beta_1$ antibody partially inhibits fibronectin-induced ureteric bud cell branching. Ureteric bud cells grown in three-dimensional collagen type I gel after 2 days in culture show no spreading or branching (*A*). Cells grown in fibronectin containing gel (50 μg/mL) show spreading and cord formation (*B*). Anti-$\alpha_3\beta_1$ antibodies partially inhibit ureteric bud cell cord and tubule formation. However, ureteric bud cells still spread and form branches (*C*).

[15]. These mice develop mesodermal defects and neural tube and vascular development aberration. Animals die prior to nephrogenesis and the latest developmental point could be examined was at gestational day 8.5 (E8.5). Early lethality of knock out mice before the development of ureteric bud as a distinct structure did not allow the study of the consequences of absence of fibronectin on ureteric bud development. Fibronectin effect on branching morphogensesis was demonstrated in embryonic salivary gland, lung, and kidney [17]. The effect of fibronectin on branching morphogenesis of salivary gland is mediated via α_5 and α_6 integrin subunits. However, the involvement of these integrins in kidney morphogenesis was not examined [17]. In the same study, kidney branching morphogenesis was inhibited by fibronectin small interfering RNA (siRNA) and anti-fibronectin and stimulated by exogenous fibronectin. Examination of the interaction between fibronectin and ureteric bud cells in three-dimensional gel culture offer a simplified system to dissect the cellular processes that fibronectin induces in ureteric bud cells with consequent cellular migration and tubule formation. In the three-dimensional gel system, anti-α_6 did not block ureteric bud cell tubule formation. We did not examine anti-α_5 since both ureteric bud and ureteric bud cells do not express α_5 integrin subunits. It is possible that there are differential organ effects of fibronectin such that α_5 mediates fibronectin effect in salivary gland and $\alpha_3\beta_1$ mediates fibronectin effect on the kidney branching morphogenesis.

Multiple genes are shown to participate in metanephric development and tubulogenesis such as bone morphogenic protein-7 (BMP-7), HGF, N-myc, epidermal growth factor (EGF), transforming growth factor-α (TGF-α), nerve growth factor (NGF), laminin, and fibronectin [24–26]. HGF-transfected mouse IMCD-3 cells exhibit scattered morphology and increased cell motility and they form branching tubules when grown in three-dimensional collagen gel. It is interesting that expression of HGF and its receptor C-met in these cells also stimulates fibronectin gene expression [16]. The extent of fibronectin expression and its effect on tubule formation of these transfected cells in three-dimensional collagen gel was not studied. Also the expression of fibronectin in Madin-Darby canine kidney (MDCK) cells treated with HGF is not known. It is possible that fibronectin is a downstream gene activation target in both MDCK cells that form tubule in response to HGF and IMCD-3 cells transfected with HGF.

It has been shown that fibronectin influences a variety of cellular processes through interaction with integrin and nonintegrin receptors with subsequent modulation of signal transduction pathways within cells. Among fibronectin receptors both $\alpha_3\beta_1$ and $\alpha_v\beta_3$ are expressed in the developing ureteric bud [27–29].

Studies that examined the effect of fibronectin on kidney branching morphogenesis are contradictory. Early studies that examined the effects of antifibronectin antibodies and fibronectin synthetic peptides on epithelial induction in organ cultures of 11.5-day-old mouse kidneys failed to show an effect on nephrogensis [15, 30]. In these studies antifibronectin antibodies have no effect on ureteric bud branching and 70 kD gelatin-binding fragment of fibronectin has no effect on DNA synthesis [30]. However, a recent study demonstrated that fibronectin causes branching morphogenesis of the salivary gland, lung, and kidney. Fibronectin siRNA inhibited

and exogenous fibronectin stimulated branching morphogenesis of embryonic kidneys grown on nucleopore filters. These observations support our findings that fibronectin induces ureteric bud cells to form cord and tubule in three-dimensional gel culture. However, these observations were made in organ culture system where contribution of other endogenous soluble or matrix components may contribute to branching morphogenesis. The absence of an effect of the 70 kD gelatin-binding fragment of fibronectin is an indication that fibronectin interacts with ureteric bud cells through other domains such as RGD peptide sequence and the absence of the effect of the gelatin-binding fragment does not exclude a biologic role for fibronectin through other active sites. Even fibronectin-null mutants have genuine differences in the severity of phenotype [15] that can be attributed to the mixed genetic background of the mice.

During inductive interaction, we and others [31] have shown that fibronectin is down-regulated in the induced area of mesenchyme. Fibronectin, however, remains associated with the basement membrane of the branching ureter [31]. We also show that cultured ureteric bud cells express fibronectin. Such findings indicate that ureteric bud epithelium is a source of fibronectin in the embryonic kidney especially fibronectin localized adjacent to ureteric bud epithelium. It was demonstrated that fibronectin expression is mainly at the constriction points of the branching ureter [17]. Our data indicate that fibronectin is expressed uniformly at the tips and stalks of branching ureter. We do not have an explanation for this discrepancy. However, the use of different antifibronectin antibody may have contributed. These data, together with the recent observation [32] that the deposition of fibronectin facilitates branching tubulogensis of MDCK (cells derived from collecting duct) strongly support a role for fibronectin as a mediator of ureteric bud tubulogenesis.

We also show that HGF, FGF, and GDNF induce ureteric bud cell cord formation in three-dimensional collagen type I gel. This effect was only seen after 4 days in three-dimensional gel culture except for HGF that induces significant effect after 2 days. Fibronectin effect was significant after 24 hours and it was many folds higher than any of the tested growth factors. The effect of the growth factors was additive to that of fibronectin suggesting that different cellular mechanisms mediate effects of growth factors.

CONCLUSION

Fibronectin induces ureteric bud cell branching and formation of cellular cords and tubules in three-dimensional gel culture through interaction with its integrin receptors and that $\alpha_3\beta_1$ integrin receptor partially mediates fibronectin cellular interaction. The significance of this in vitro observation is supported by the recent finding that kidney branching morphogenesis is inhibited by fibronectin siRNA and antifibronectin and stimulated by exogenous fibronectin [17]. It remains necessary to study the intracellular signal transduction pathways that are activated in response to fibronectin integrin receptors interactions. It is also important to study the cytoskeletal assemblies that likely lead to signaling complexes, including focal adhesion kinase (FAK), phosphatidylinositol-3-kinase (PI-3K), and mitogen-activated protein kinase (MAPK) activation. MAPK activation is likely to stimulate genes such as metalloproteinases which in turn may facilitate ureteric bud cells migration and cord/tubule formation.

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